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Influence of Plant Growth Regulators on *in Vitro* Seed Germination and Seedling Development of *Digitalis purpurea* L.

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ABSTRACT

Digitalis purpurea L. (foxglove; Scrophulariaceae) is an herbaceous medicinally important cardiac glycoside-producing plant. The aim of the present study was to access the seed viability and influence of plant growth regulators on *in vitro* seed germination and seedling development. The 2,3,5-triphenyl tetrazolium chloride (TTC) test showed that 100% of seeds were viable while a direct germination test in soil and in Petri dishes showed only about 20% germination ability. The surface-sterilized seeds were cultured *in vitro* on Murashige and Skoog (MS) medium containing 3% sucrose, 0.8% agar and different concentrations (0 to 15.0 µM) of cytokinins (6-benzyladenine - BA; kinetin - Kin and thidiazuron - TDZ) and auxins (α-naphthaleneacetic acid - NAA; indole-3-acetic acid - IAA and 2,4-dichlorophenoxy acetic acid - 2,4-D) alone and in combination. Addition of all types and concentrations of cytokinins and auxins stimulated the rate and percentage of seed germination. Significantly higher seed germination (65.5 ± 1.2% and 63.1 ± 3.2%) was observed on MS medium containing 10.0 µM BA and Kin, respectively than control (16.7 ± 3.1%). Addition of 10.0 µM IAA in the MS medium was most effective for significantly highest (81.0 ± 3.1%) germination percentage. This was evident by significantly higher germination speed (GS; 2.70 ± 0.1), germination value (GV; 31.3 ± 2.4) and vigor index (VI; 259.1 ± 10.1) on MS medium fortified with 10.0 µM IAA as compared with control (GS: 0.56 ± 0.1; GV: 01.4 ± 0.5 and VI: 50.0 ± 09.4). Addition of cytokinins and auxins to the culture medium significantly increased the growth of seedlings. The protocol developed in the present study can be used for large-scale seedling formation and biomass production of this important medicinal plant. It also used to obtain sterile and uniform starting material for various *in vitro* studies for the improvement of this plant.

Keywords: dormancy, germination speed, germination value, Scrophulariaceae, vigor index

Abbreviations: 2,4-D, 2,4-dichlorophenoxy acetic acid; **BA**, 6-benzyladenine; **CGP**, cumulative germination percentage; **FGP**, final germination percentage; **GS**, germination speed; **GV**, germination value; **IAA**, indole-3-acetic acid; **Kin**, kinetin; **MS**, Murashige and Skoog; **NAA**, α-naphthaleneacetic acid; **PGR**, plant grwoth regulator; **SDW**, sterilized distilled water; **VI**, vigor index; **TDZ**, thidiazuron; **TTC**, 2,3,5-triphenyltetrazolium chloride

INTRODUCTION

Digitalis purpurea L. (foxglove; Scrophulariaceae) is an herbaceous biennial or perennial with erect stems of 3 to 6 feet in height. Leaves grow as a rosette during first year of growth. Lower leaves grow about 12 inches long and 2 inches wide, soft hairy above with toothed margin. During second year of growth, plant produces a leafy stock bearing a tall spike of bell shaped pink colored flowers with dark spots on lower inside surface. Fruits are ovoid capsules with many minute seeds (Harris 2000; Whitson *et al*. 2000).

A group of pharmacologically active compounds (cardiac glycosides) extracted from the leaves of two years grown plants namely digitoxin, digoxin and lanatoside C are best known products to strengthen cardiac diffusion and to regulate heart rhythm (Navarro *et al*. 2000; Pérez-Bermúdez *et al*. 2010; Sharma and Purkait 2012). It is used to increase the contractility of cardiac muscles and as an antiarrhythmic agent to control the heart rate. It works by inhibiting the sodium-potassium ATPase activity results in increased intracellular concentration of sodium. The increase in intracellular sodium increases calcium passively by decreasing the sodium-calcium exchanger in sarcolemma. The increased intracellular calcium gives a positive inotropic effect (Rahimtoola and Tak 1996; Xie and Askari 2002; Mohammadi *et al*. 2003; López-Lázaro 2007; Kuate *et al*. 2008; Wu *et al*. 2012). Treatment with cardiac glycosides is still the only safe inotropic drug for oral use which improves haemodynamics in patients with a compromised cardiac function (Schwinger *et al*. 2003; Pérez-Alonso *et al*. 2009). Recent research supports the potential of *Digitalis* cardiac glycosides for the treatment of several types of cancer (Haux 1999; Stenkvist 2001; Haux *et al*. 2001; López-Lázaro *et al*. 2003; López-Lázaro 2007; Sharma and Purkait 2012; Wu *et al*. 2012). Entire plant including leaves, roots and seeds is poisonous and contain several deadly physiologically and chemically related cardiac and steroidal glycosides. Overdose of *Digitalis* causes anorexia, nausea, vomiting, diarrhea, headache, drowsiness, disorientation, hallucination and xanthopsia (Budavari *et al*. 1989).

The propagation of plants by seeds is comparatively easy, fast and reliable. Seed can be considered as the starting structure in the life of seed plants. Successful seed germination depends on numerous internal and external factors. Seed germination involves the protrusion of embryonic axis from the seed to resume plant growth (Finkelstein *et al*. 2008; Park *et al*. 2011). However, many seeds exhibit dormancy and fail to germinate even in favourable conditions. Seed germination is influenced by internal factors controlling dormancy, including phytohormones inducing dormancy (ABA), and seed coat factors (Baskin and Baskin 1998; Holdsworth *et al*. 2008; Linkies and Leubner-Metzger 2012). Depending on the plant species and type of dormancy, various methods like scarification, stratification, removal of inhibitor and treatment with growth regulators are used to break dormancy (Baskin and Baskin 1998; Hidayati *et al*. 2012).

Phytohormones represent a group of organic molecules

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that are produced by plant tissues and translocated to some other tissue where they influence many diverse developmental processes (McCourt 1999; Kucera *et al*. 2005; Bakrim *et al*. 2007). Phytohormones regulate and integrate the overall growth, development and reproduction in plants by acting as chemical messengers for the communication among cells, tissues and organs (Kucera *et al*. 2005). Specific endogenous hormones and its levels are directly involved in the control of seed development, dormancy and germination (Hartman *et al*. 1997). Correlations of concentration of endogenous level of hormone with specific developmental stages, effects of applied hormones, and the relationship with metabolic activities suggests an involvement of hormones in these metabolic activities (Pedroza-Manrique *et al*. 2005).

Natural regeneration of *Digitalis purpurea* is by seeds and seeds remain viable in soil for at least five years (Harris 2000). Nursery germination of *D. purpurea* is poor and non-predictable due to specific temperature requirement for germination and specific storage conditions of seeds to maintain the viability (Butler *et al*. 2009). Multiplication of medicinal plants is essential for biomass production and there is clear need to formulate a procedure that will facilitate quick and reliable germination. Biotechnological tools can be applied to achieve rapid multiplication of many medicinal plants including forest tree species in a short time (Nehra *et al*. 2005; Nikam and Barmukh 2009). In the present investigation, we describe a protocol for an *in vitro* technique to induce uniform and faster germination of *D. purpurea* for mass propagation.

MATERIALS AND METHODS

Seed source, surface sterilization and seed viability

The seeds of *Digitalis purpurea* L. were collected from the plants grown at Herbal Research and Development Institute (HRDI), Mandal, Chamoli, Uttarakhand, India. The seeds were stored in air tight plastic bottles in dark at room temperature. The seeds were surface-sterilized using 0.1% (w/v) aqueous mercuric chloride (Qualigens, Mumbai, India) solution for 5 min followed by washing with sterilized distilled water (SDW) for 5 times. Then the surface-sterilized seeds were used for the treatments and germination trials *in vitro*. All the chemicals and reagents were purchased from Hi Media, Mumbai, India and plant growth regulators were procured from Sigma-Aldrich (Bangalore, India).

The viability of seeds was tested by the 2,3,5-triphenyltetrazolium chloride (TTC) test (Hartman *et al*. 1997) as described by Ahire *et al*. (2009). About 50 seeds were soaked in 0.5% (w/v) TTC in 50 mM sodium phosphate buffer (pH 7.0) for 24 h in the dark. Then the seeds were washed three times with SDW and the embryos were dissected out by removal of seed coat and cotyledons using needle and forceps and observed for change in color to red. The seeds were also sown in moistened soil in earthen pots and Petri dishes (10 cm diameter, Axygen, New Delhi, India) containing two layers of moist germination paper (1 mm thick, Modern Paper Ltd., Pune, India) and observed for viability and germination.

Culture medium and culture conditions

Five surface-sterilized seeds were inoculated in each Borosilicate glass test tube (25 × 150 mm, Borosil, Mumbai, India) containing MS (Murashige and Skoog 1962) medium supplemented with 30 g l^{-1} sucrose and different concentrations of cytokinins (6-benzyladenine - BA; kinetin - Kin and thidiazuron - TDZ; 00.0-15.0 µM) and auxins (α-naphthalene acetic acid - NAA; indole-3-acetic acid - IAA and 2,4-dichlorophenoxy acetic acid - 2,4-D; 00.0-15.0 µM) individually or in combination. The seeds inoculated on MS medium without fortification of plant growth regulators served as control. The pH of the medium was adjusted to 5.8 and solidified with 0.8% (w/v) agar (Hi Media, India) prior to autoclaving at 121°C for 15 min. The cultures were incubated under controlled conditions such as 25 ± 2°C temperature, 60 ± 10% relative humidity and 8 h photoperiod (PFD = 40 µmol m^{-2} s^{-1}) provided by white fluorescent tubes (Philips, Kolkata, India).

Data collection on germination

The cultures were observed daily and the data on daily seed germination was collected until the completion of germination (maximum up to 30 days). The seeds with 0.5 mm or more radical growth occur were counted as germinated seeds. The final germination percentage (FGP) was calculated from the total seeds that germinated on the day of completion.

Other germination parameters such as germination speed (GS) was calculated using the formula as described by Aldhous (1972): GS = FGP/Day of completion of germination.

Germination values (GV) were calculated using the formula described by Djavanshir and Pourbeik (1976):

$$GV = (\Sigma DGs / N) GP / 10$$

where, GV = germination value, GP = germination percentage at the end of the test, DGs = daily germination speed obtained by dividing the cumulative germination percentage by the number of days since sowing, Σ DGs = the total germination obtained by adding every DGs value obtained from the daily counts, N = the total number of daily counts, starting from the date of first germination, 10 = constant.

Data collection on growth parameters

Different growth parameters were observed on the day of completion of the germination. Seedling vigor Index (SVI) was calculated as per the recommendations of ISTA (1976):

SVI = Germination percent × Shoot length.

Root length and shoot length of the seedlings were recorded and root to shoot ratio was calculated. Fresh weight (FW) of seedlings was recorded and dried in an oven at 60°C until constant weight and then dry weight (DW) of seedlings were recorded. Moisture content of seedlings was calculated using formula: Fresh weight - Dry weight/Fresh weight × 100. All the observations were recorded on the 30^{th} days of incubation.

Seedling development

In vitro germinated seedlings were transferred to earthen pots containing moist garden soil. The pots with plantlets were maintained in a shade-net (Green Net India Pvt. Ltd., Ahmadabad, India) house (equipped with the net that can cut 50% of the incident light) in the Botanic Garden of the Department of Botany, University of Pune, Pune. Watering to the seedlings was carried out as and when required to maintain the moisture.

Statistical analysis of data

The experiment was carried out in a completely randomized design with seven replicates. In each treatment 5 seeds were inoculated in 10 test tubes thus for each treatment 50 seeds were used and each treatment was repeated three times. Data were analyzed by analysis of variance (ANOVA) to detect significant differences between means. Means differing significantly were compared using Duncan's multiple range test (DMRT) at the 5% probability level using statistical software program SPSS version 9.0. Variability in data has been expressed otherwise as mean ± standard error (SE).

RESULTS AND DISCUSSION

Seed viability

Seed viability indicates the capability of seeds to germinate and produce normal seedlings under suitable germination conditions (Copeland and McDonald 2001). It has been known that three factors; temperature, seed moisture content and oxygen pressure are most important for viability

and longevity of seeds in storage. Total germination depends largely on the viability and vigor of the seeds used (Harrington 1972; Ellis *et al*. 1993; Hay and Probert 1995). In the present investigation, viability of the seeds of *Digitalis purpurea* was checked using TTC test. The seeds were soaked in TTC solution for 24 h in the dark. All the embryos including cotyledons were stained red. These results showed that the TTC dye which was in contact with embryo got reduced and stained the embryo into red color. Viability tests in conjunction with germination experiments explains better to define the reproductive potential of seeds over time (Drewitz and DiTomaso 2004; Conklin and Sellmer 2009). It indicated that the 100% seeds were viable. But the seeds sowed in the petri plate and soil showed about 20% germination during the incubation period. The variation in viability testing and soil germination is might be due to the presence of dormancy in the seeds. The results indicate that the seeds of *D. purpurea* possess some kind of dormancy.

Similar studies were carried on viability testing of the seeds using TTC test in *Uraria picta* showed 100% viability (Ahire *et al*. 2009); seeds of *Bunium persicum* showed 93% viability (Sharma and Sharma 2010). Ghane *et al*. (2010) tested the seed viability of seeds of *Indigofera glandulosa* using TTC test showed 100% seeds were found viable. Seeds of *Astragalus membranaceus* showed 62.5% viability, whereas seeds of *Magnolia officinalis* and *Panax notoginseng* exhibited 100% viability (Zhou *et al*. 2012). In the present investigation, actual germination in soil and viability of seeds tested using TTC test showed variation. Similarly, Masumoto and Ito (2010) reported that some of the mericarps stored for more than 10 years were stained red by TTC even though the germination rate was 0% in the seed of *Perilla frutescens*.

Effect of plant growth regulators on seed germination

Surface-sterilized seeds of *D. purpurea* were inoculated on MS medium supplemented with different (00.0 – 15.0 µM) concentrations of cytokinins (BA, Kin and TDZ) and auxins (NAA, IAA and 2,4-D) alone and in combination. In control (MS medium without fortification of PGR), about 16.7 ± 3.1% FGP (**Table 1**) up to the incubation period. Best germination for *Sarracenia leucophylla* and *S. purpurea* occurred on one-third strength Murashige and Skoog (MS) salts, whereas *S. oreophila* germinated best on one-sixth strength MS salts. Adjustment of pH to 4.5 to simulate a bog environment further increased germination for *S. leucophylla* (Northcutt *et al*. 2012). Seeds of *Pinus peuce* did not showed significant difference in germination in Gresshoff and Doy (GD; 1972) medium without plant growth regulators (PGR) and GD medium fortified with different PGR's (Stojičić *et al*. 2012). About 88% of seeds germinated on GD medium without PGR. In the present study, seeds showed the sign of germination after 15 days of inoculation. Whereas, the TTC test showed 100% seed viability. The seeds inoculated on MS medium showed 16.7 ± 3.1% (**Table 1**) germination similar to the germination occurred in the Petri dishes or in soil. These results showed that there is some kind of dormancy exists in the seeds of *Digitalis purpurea*.

The seed development, dormancy and germination are controlled by specific endogenous growth promoting and inhibiting compounds (Hartman *et al*. 1997) and there is a correlation of hormone concentration with specific developmental stages, effects of applied hormones, and the relationship of hormones to metabolic activities (Pedroza-Manrique *et al*. 2005). Cytokinins and auxins were applied to stimulate subsequent regeneration in seedlings. However, an unexpected effect was noted with respect to seed germination. In the present investigation, all cytokinins and auxins used were able to accelerate the germination rate and increase the FGP. PGRs involved in the regulation of various processes of plant growth and development (Nikolić *et al*. 2006). Among their multiple activities, the effects of cytokinins on

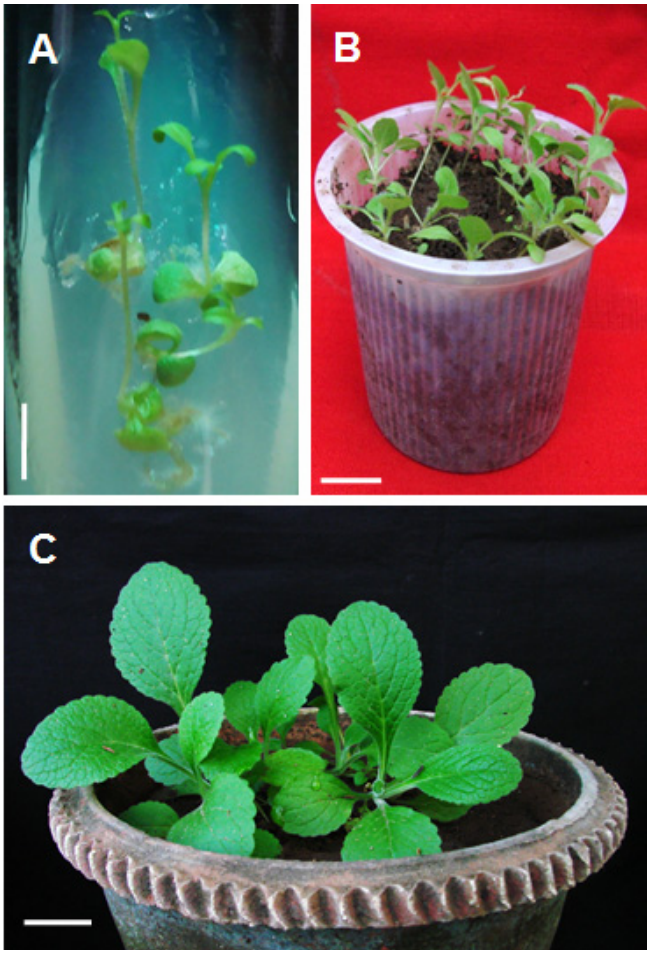

Fig. 1 *In vitro* seed germination and seedling development in *Digitalis purpurea* L. (**A**) *In vitro* germinated seedlings; (**B**) Germinated seedlings transferred to soil in plastic container after 28 days of *in vitro* growth; (**C**) Three month old plantlets in earthen pots.

seed germination stand apart from their role in shoot morphogenesis. Recent researches showed cytokinins have shown their very active metabolism in all phases of germination, from imbibition to radicle emergence and the start of seedling establishment (Chiwocha *et al*. 2005; Stirk *et al*. 2005; Nikolić *et al*. 2006).

Among the different types and concentrations of cytokinins BA (65.5 ± 1.2% FGP) showed better results for germination of seeds followed by Kin (63.1 ± 3.2% FGP) at 10.0 µM. At this concentration of BA and Kin other germination parameters such as GS (2.18 ± 0.1, 2.10 ± 0.1), GV (20.4 ± 0.7, 19.1 ± 1.9) and VI (320.9 ± 05.8, 258.7 ± 12.9), respectively (**Table 1**) was also higher over control and other types and concentrations of cytokinins. TDZ was also found effective for enhanced germination at lower concentrations. About 41.7 ± 3.2% FGP (**Table 1**) was observed at 2.5 µM TDZ, increasing concentration of TDZ was toxic for seeds germination wherein decreased in germination was observed with significant decrease in other germination parameters such as GS, GV and VI (**Table 1**). In contrast, Babiker *et al*. (1994) reported the promotion in germination of seeds treated with TDZ in *Striga asiatica*. In promoting seed germination, ZEA, TDZ, and BA occupy the first place, with inconsistent differences between them as reported by Nikolić *et al*. (2006) in *Lotus corniculatus*. Exogenous application of cytokinins has different types of effects on seed germination in different species (Nikolić *et al*. 2006). Whereas, cytokinins did not affect seed germination in seeds of several large-seeded grain legumes (Malik and Saxena 1992). The promotive effect of cytokinins on seed germination is mostly related to the alleviation of stress factors. Among the different cytokinins, Kin was one of the PGR that alleviate both innate and salinity-induced seed

Table 1 Effect of cytokinins on *in vitro* seed germination in *Digitalis purpurea*.

Cytokinins (µM)	Germination (%)	GS	GV	VI
BA				
00.0	16.7 ± 3.1 fg	0.56 ± 0.1 h	01.4 ± 0.5 ef	050.0 ± 09.4 h
02.5	21.4 ± 2.1 fg	0.71 ± 0.1 gh	02.2 ± 0.4 ef	094.3 ± 09.1 g
05.0	26.2 ± 2.4 ef	0.87 ± 0.1 fg	03.3 ± 0.6 de	136.2 ± 12.4 e
07.5	41.7 ± 1.2 d	1.39 ± 0.1 e	08.3 ± 0.5 c	212.5 ± 06.1 cd
10.0	65.5 ± 1.2 a	2.18 ± 0.1 a	20.4 ± 0.7 a	320.9 ± 05.8 a
15.0	54.8 ± 1.2 b	1.83 ± 0.1 b	14.3 ± 0.6 b	235.5 ± 05.1 bc
Kin				
02.5	32.2 ± 3.6 e	1.07 ± 0.1 f	05.0 ± 1.0 d	118.9 ± 13.2 efg
05.0	40.5 ± 2.4 d	1.35 ± 0.1 e	07.9 ± 0.9 c	198.4 ± 11.7 d
07.5	46.4 ± 2.1 cd	1.55 ± 0.1 cd	10.3 ± 0.9 c	232.2 ± 10.3 bc
10.0	63.1 ± 3.2 a	2.10 ± 0.1 a	19.1 ± 1.9 a	258.7 ± 12.9 b
15.0	52.4 ± 2.4 bc	1.75 ± 0.1 bc	13.1 ± 1.2 b	199.1 ± 09.1 d
TDZ				
02.5	41.7 ± 3.2 d	1.39 ± 0.1 e	08.4 ± 1.2 c	133.3 ± 10.1 ef
05.0	29.8 ± 2.3 e	0.99 ± 0.1 f	04.3 ± 0.6 de	113.1 ± 09.1 efg
07.5	26.2 ± 1.2 ef	0.87 ± 0.1 fg	03.3 ± 0.3 de	104.8 ± 04.8 fg
10.0	17.9 ± 2.1 fg	0.60 ± 0.1 h	01.6 ± 0.4 ef	055.4 ± 06.4 h
15.0	08.3 ± 1.2 h	0.28 ± 0.1 i	00.3 ± 0.1 f	023.4 ± 03.3 i

Data presented in the table are mean ± SE (standard error) scored at 30 days of inoculation from 10 test tubes per treatment with 5 seeds each and repeated thrice. Means followed by same letters within columns are not significantly different at $P \leq 0.05$ level by DMRT. GS: Germination speed; GV: Germination value; VI: Vigor index

Table 2 Effect of cytokinins on seedling growth and biomass production in *Digitalis purpurea.*

Cytokinins (µM)	Shoot length (cm)	Root length (cm)	Root/shoot ratio	Fresh weight of seedlings (g)	Dry weight of seedlings (g)	Moisture content (%)
BA						
00.0	3.0 ± 0.1 h	1.8 ± 0.1 h	0.60 ± 0.1 g	2.6 ± 0.1 fgh	0.26 ± 0.02 h	90.0 ± 0.1 a
02.5	4.4 ± 0.1 c	3.0 ± 0.1 b	0.68 ± 0.1 e	3.7 ± 0.1 bc	0.37 ± 0.03 b	90.0 ± 0.1 a
05.0	5.2 ± 0.1 a	3.3 ± 0.1 a	0.63 ± 0.1 f	4.3 ± 0.1 a	0.39 ± 0.07 a	90.9 ± 0.1 a
07.5	5.1 ± 0.0 ab	3.0 ± 0.1 ab	0.59 ± 0.2 g	3.6 ± 0.1 ab	0.36 ± 0.02 b	90.0 ± 0.1 a
10.0	4.9 ± 0.0 b	2.7 ± 0.0 cd	0.55 ± 0.1 h	3.2 ± 0.2 de	0.32 ± 0.03 d	90.0 ± 0.1 a
15.0	4.3 ± 0.1 cd	2.5 ± 0.0 de	0.58 ± 0.1 g	3.4 ± 0.0 cde	0.33 ± 0.02 c	90.3 ± 0.1 a
Kin						
02.5	3.7 ± 0.2 g	2.7 ± 0.1 cd	0.73 ± 0.1 b	3.0 ± 0.1 efg	0.30 ± 0.01 f	90.0 ± 0.1 a
05.0	4.9 ± 0.1 b	2.9 ± 0.1 bc	0.59 ± 0.1 g	3.3 ± 0.1 cde	0.34 ± 0.01 c	89.7 ± 0.1 b
07.5	5.0 ± 0.1 ab	2.5 ± 0.0 de	0.50 ± 0.1 i	3.6 ± 0.0 bcd	0.37 ± 0.03 b	89.7 ± 0.1 b
10.0	4.1 ± 0.0 de	2.3 ± 0.1 ef	0.56 ± 0.1 h	3.2 ± 0.1 de	0.32 ± 0.01 cd	90.0 ± 0.1 a
15.0	3.8 ± 0.1 fg	2.1 ± 0.0 fg	0.55 ± 0.0 h	2.9 ± 0.2 gh	0.29 ± 0.02 fg	90.0 ± 0.1 a
TDZ						
02.5	3.2 ± 0.1 h	2.3 ± 0.0 ef	0.72 ± 0.1 bc	2.7 ± 0.1 fgh	0.28 ± 0.05 gh	89.6 ± 0.1 b
05.0	3.8 ± 0.1 fg	2.7 ± 0.1 cd	0.71 ± 0.2 cd	3.0 ± 0.0 h	0.30 ± 0.04 ef	90.0 ± 0.1 a
07.5	4.0 ± 0.1 ef	3.1 ± 0.1 ab	0.78 ± 0.1 a	3.1 ± 0.1 ef	0.31 ± 0.01 de	90.0 ± 0.1 a
10.0	3.1 ± 0.1 h	2.1 ± 0.0 fg	0.68 ± 0.1 e	2.7 ± 0.1 fgh	0.28 ± 0.03 g	89.6 ± 0.1 b
15.0	2.8 ± 0.1 i	2.0 ± 0.2 g	0.71 ± 0.2 d	2.4 ± 0.1 h	0.27 ± 0.02 h	88.8 ± 0.1 c

Data presented in the table are mean ± SE (standard error) scored at 30 days of inoculation from 10 test tubes per treatment with 5 seeds each and repeated thrice. Means followed by same letters within columns are not significantly different at $P \leq 0.05$ level by DMRT.

Table 3 Effect of auxins on *in vitro* seed germination in *Digitalis purpurea.*

Auxins (µM)	Germination (%)	GS	GV	VI
IAA				
02.5	44.1 ± 3.1 ef	1.47 ± 0.1 ef	09.3 ± 1.3 fgh	185.0 ± 13.2 d
05.0	57.2 ± 2.1 c	1.91 ± 0.1 c	15.6 ± 1.1 d	228.6 ± 08.2 b
07.5	69.1 ± 2.4 b	2.30 ± 0.1 b	22.8 ± 1.5 c	269.3 ± 09.3 a
10.0	81.0 ± 3.1 a	2.70 ± 0.1 a	31.3 ± 2.4 a	259.1 ± 10.1 a
15.0	76.2 ± 2.4 ab	2.54 ± 0.1 a	27.7 ± 1.7 b	228.6 ± 07.2 bc
NAA				
02.5	14.3 ± 2.1 j	0.48 ± 0.1 j	01.0 ± 0.3 k	044.3 ± 06.4 i
05.0	31.0 ± 1.2 hi	1.03 ± 0.1 h	04.6 ± 0.3 ij	117.6 ± 04.5 fg
07.5	52.4 ± 1.2 cd	1.75 ± 0.1 cd	13.1 ± 0.6 de	209.5 ± 04.8 c
10.0	45.2 ± 1.2 def	1.51 ± 0.1 ef	09.8 ± 0.5 efg	171.9 ± 04.5 d
15.0	35.7 ± 2.1 gh	1.19 ± 0.1 gh	06.1 ± 0.7 hi	128.6 ± 07.4 ef
2,4-D				
02.5	23.8 ± 2.4 i	0.79 ± 0.1 i	02.8 ± 0.6 jk	090.5 ± 09.1 h
05.0	48.8 ± 1.2 de	1.63 ± 0.1 de	11.4 ± 0.5 ef	170.8 ± 04.2 d
07.5	46.4 ± 2.1 cd	1.55 ± 0.1 def	10.3 ± 0.9 efg	143.9 ± 06.4 e
10.0	40.5 ± 1.4 fg	1.35 ± 0.1 fg	07.9 ± 0.9 ghi	121.4 ± 07.1 fg
15.0	33.3 ± 1.2 gh	1.11 ± 0.1 h	05.3 ± 0.4 ij	103.4 ± 03.7 gh

Data presented in the table are mean ± SE (standard error) scored at 30 days of inoculation from 10 test tubes per treatment with 5 seeds each and repeated thrice. Means followed by same letters within columns are not significantly different at $P \leq 0.05$ level by DMRT. GS: Germination speed; GV: Germination value; VI: Vigor index

Table 4 Effect of auxins on seedling growth and biomass production in *Digitalis purpurea*.

Auxins (µM)	Shoot length (cm)	Root length (cm)	Root/shoot ratio	Fresh weight of seedlings (g)	Dry weight of seedlings (g)	Moisture content (%)
IAA						
02.5	4.2 ± 0.1 d	3.8 ± 0.2 gh	0.90 ± 0.2 g	3.1 ± 0.0 e	0.29 ± 0.03 fg	90.6 ± 0.1 ab
05.0	4.0 ± 0.1 ab	3.9 ± 0.1 de	0.98 ± 0.1 ef	2.7 ± 0.0 de	0.28 ± 0.01 cde	89.6 ± 0.1 b
07.5	3.9 ± 0.1 ab	4.4 ± 0.0 bc	1.13 ± 0.1 c	3.1 ± 0.1 ab	0.29 ± 0.05 abc	90.6 ± 0.1 ab
10.0	3.2 ± 0.1 ab	4.8 ± 0.1 def	1.50 ± 0.1 a	3.3 ± 0.1 ab	0.31 ± 0.08 ab	90.6 ± 0.1 ab
15.0	3.0 ± 0.1 bc	4.3 ± 0.1 gh	1.43 ± 0.1 b	3.0 ± 0.2 cd	0.25 ± 0.05 abcd	91.7 ± 0.2 a
NAA						
02.5	3.1 ± 0.2 a	3.2 ± 0.1 de	1.03 ± 0.1 ef	2.5 ± 0.0 ab	0.26 ± 0.03 bcd	89.6 ± 0.1 b
05.0	3.8 ± 0.3 ab	3.8 ± 0.1 cd	1.00 ± 0.1 ef	2.7 ± 0.1 de	0.28 ± 0.01 cde	89.6 ± 0.1 b
07.5	4.0 ± 0.1 ab	4.2 ± 0.1 b	1.05 ± 0.1 de	3.1 ± 0.1 ab	0.29 ± 0.02 abc	90.6 ± 0.1 ab
10.0	3.8 ± 0.1 cd	3.7 ± 0.2 a	0.97 ± 0.2 f	3.1 ± 0.1 a	0.30 ± 0.04 a	90.3 ± 0.1 ab
15.0	3.6 ± 0.0 d	3.2 ± 0.1 b	0.89 ± 0.1 g	2.8 ± 0.1 bc	0.29 ± 0.05 g	89.6 ± 0.1 b
2,4-D						
02.5	3.8 ± 0.1 ab	3.2 ± 0.1 gh	0.84 ± 0.1 g	2.6 ± 0.1 de	0.28 ± 0.03 de	89.2 ± 0.1 b
05.0	3.5 ± 0.1 cd	3.5 ± 0.1 efg	1.00 ± 0.1 ef	3.0 ± 0.0 bc	0.29 ± 0.01 abcd	90.3 ± 0.1 ab
07.5	3.1 ± 0.1 d	3.4 ± 0.1 fgh	1.10 ± 0.1 cd	3.1 ± 0.1 ab	0.30 ± 0.01 ab	90.3 ± 0.1 ab
10.0	3.0 ± 0.1 d	3.1 ± 0.1 h	1.03 ± 0.1 ef	3.0 ± 0.1 bc	0.27 ± 0.02 ef	91.0 ± 0.1 ab
15.0	3.1 ± 0.0 d	2.7 ± 0.1 i	0.87 ± 0.1 g	2.6 ± 0.1 de	0.28 ± 0.01 cde	89.2 ± 0.1 b

Data presented in the table are mean ± SE (standard error) scored at 30 days of inoculation from 10 test tubes per treatment with 5 seeds each and repeated thrice. Means followed by same letters within columns are not significantly different at $P \leq 0.05$ level by DMRT.

dormancy in many halophytes (Khan and Ungar 1997; Khan *et al*. 2004). Kin was also reported as possible protections of cell membranes against oxidative stress which may be resulted in prolong the viability of recalcitrant seeds (Chaitanya and Naithani 1998). The promotive effects on seed germination of several cytokinins, such as BA, Kin, TDZ and zeatin were reported in orchids like *Cypripedium macranthos* (Miyoshi and Mii 1998), *Habenaria macroceratitis* (Stewart and Kane 2006) and *Calanthe* hybrids (Shin *et al*. 2011). Supplementation of both NAA and BA in the medium stimulated seed germination in endangered terrestrial orchid *Calanthe tricarinata*, but BA was more effective than NAA (Godo *et al*. 2010). GD medium fortified with different concentrations of BA and Kin showed germination of seeds in the range of 72-80% in *Pinus peuce* (Stojičić *et al*. 2012).

Among the different types and concentrations of auxins, IAA at 10.0 µM was found to be effective for enhanced seed germination. At this concentration about 81.0 ± 3.1% FGP (**Table 3; Fig. 1A**) was observed and it was higher than any other PGR used. GS (2.70 ± 0.1) and GV (31.3 ± 2.4) was also found higher (**Table 3**) than any other treatment of PGR. Higher germination was observed in IAA containing media followed by 7.5 µM NAA where about 52.4 ± 1.2% FGP was observed. Different concentrations of 2,4-D was also enhancing the germination which was in the range of 23-49% germination (**Table 3**). The VI was also found to be higher 259.1 ± 10.1 (**Table 3**) but it was lower than the BA treated seeds. Auxins had a slight promotive effect for *in vitro* seed germination in *Paphiopedilum ciliolare* Pfitz. (Pierik *et al*. 1988) and *Paphiopedilum wardii* Sumerh. (Zeng *et al*. 2012). Similar to the results obtained in the present investigation, promotive effect of IAA in seed germination of *Comparettia falcata* was reported by Pedroza-Manrique *et al*. (2005). IAA can be oxidized non-enzymatically when exposed to light and its photodestruction may be promoted by plant pigments (Taiz and Zeiger 1998). Moreover, synthetic auxins are not destroyed by IAA oxidases, so those auxins persist in plants much longer than does IAA (Salisbury and Ross 2007).

The hormonal control of germination involves a balance between the stimulating and inhibitory components of seeds (Thomas 1980; Pierik 1987; Pedroza-Manrique *et al*. 2005). In the present study, combination treatment of cytokinins and auxins inhibits the germination and resulted into slight swelling and callus formation (data not shown). Combination treatments were not useful for enhanced seed germination. Similarly, in some orchids, cytokinins-auxin (Kin-auxin) combination is not as stimulating as compared with a medium containing only Kin (Martin 2003). In contrast to these results, Pedroza-Manrique *et al*. (2005) reported the promotive effect for seed germination in *Comparettia falcata* using IAA in combination with Kin. Vejsadová (2006) also reported the promotive effect of IAA in combination with zeatin for seed germination in terrestrial orchid species. Plant hormones are signal molecules produced within the plant, and occur in extremely low concentrations and it significantly influenced the germination percentage and days to germination in *Jatropha curcus* DARL-2 as compared to untreated control (Maya *et al*. 2010). Highest germination percentage was observed in the seeds treated with 2.0 mg/l IBA (Maya *et al*. 2010). Yarnia and Tabrizi (2012) reported the priming of onion seeds with IAA showed maximum percent germination as compared with GA_3 and Kin. Highest seed germination (90%) was reported in 500 ppm Kin treated seeds on MS medium in *Bixa orellana* followed by IAA- and tricontenol-treated seeds showed maximum germination (Castello *et al*. 2012). Mahajan *et al*. (2012) reported maximum seed germination (63.89%) on MS medium supplemented with 0.5 mg/l IBA for fresh *Lisianthus* seeds. GD medium fortified with different concentrations of NAA and IBA showed seed germination in the range of 77-88% in *Pinus peuce* (Stojičić *et al*. 2012).

Seedling development

In the present investigation, the shoot length and root length of germinated seedlings of *D. purpurea* increases up to 5.0 µM of cytokinins (BA, Kin and TDZ) and decreases thereby (**Table 2**). Among cytokinins, maximum seed germination was observed at 10.0 µM each of BA and Kin but the growth parameters such as shoot length, root length, seedling FW and DW were significantly lower as compared with 5.0 µM each of BA and Kin. Similar trend was also observed in seeds germinated on auxins (IAA, NAA and 2,4-D) containing medium (**Table 4**). But these parameters were significantly higher as compared with the control. The seedlings obtained were transferred to the plastic containers (**Fig. 1B**) containing garden soil and maintained in controlled conditions for 30 days. The well acclimatized plantlets were transferred to earthen pots (**Fig. 1C**) containing garden soil after 30 days showed 100% survival under glass house conditions for about two years, but the plantlets did not showed any sign of flowering. The shoot length, fresh weight and dry weights of seedlings were found to be higher in the seedlings obtained on cytokinins containing medium (**Table 2**) as compared with the auxins containing medium (**Table 4**). But it was higher in cytokinins and auxins treatments as compared with the control (**Tables 2, 4**). The root length was found to be higher in the auxins

containing medium as compared with respective concentrations of cytokinins. Generally, auxins stimulate root formation and cytokinins enhance shoot development and cell division. In the present investigation similar trend was observed. Seedling development was better in hormone-containing medium than on medium lacking hormones. Similarly, shoot growth was significantly higher in the presence of IAA in seedlings of *Dactylorhiza maculata* subsp. *maculata* than the control. In *D. maculata* and *D. incarnata*, root growth was significantly stimulated by NAA. The cytokinin zeatin stimulated shoot growth but not root growth or the number of leaves. Roots were stimulated in the presence of NAA and the cytokinin BA (Vejsadová 2006). A similar promotive effect of plant growth hormones on seedling growth of *Vigna mungo* (black gram) and *Macrotyloma uniflorum* (horse gram) were reported by Chauhan *et al*. (2009). The best medium for growth and development of *Elaeis guineensis* Jacq. var. *dura* plantlets was MS medium supplemented with 0.1 mg/L PGR (GA_3, BA and NAA) and 2 g/L AC, which significantly increased plantlet height (9.4 cm) as well as root length (4.4 cm) (Suranthran *et al*. 2011). PGRs enhanced the seed germination and radical length to different degrees in *Cyperus esculentus* (Shen *et al*. 2011).

CONCLUSION

In conclusion, the results presented here show that it is possible to improve the production of *Digitalis purpurea* plants *in vitro*. The TTC test showed 100% seed viability but the germination test in soil and Petri dishes showed only about 20% germination. The seeds might have chemical dormancy which could be broken using a PGR treatment. The addition of cytokinins and auxins to the growth medium substantially improved germination and seedling growth. The addition of IAA showed about 80% germination which could be useful for large-scale seedling formation *in vitro*. The seedlings developed well under glasshouse conditions in earthen pots containing garden soil but did not flower.

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